Supplementary Information

Two-color lateral flow assay for multiplex detection of causative agents behind acute febrile illnesses

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Blue and Red latex based detection probe preparation

The latex based detection probes used in the study were prepared by labelling the anti-IgG antibodies (goat anti-rabbit IgG secondary antibody, γ-chain specific; Jackson ImmunoResearch Laboratories, Inc.) with Blue latex beads (LATEX conjugation kit – 400nm Blue; Innova Biosciences Ltd.), and anti-IgM antibodies (goat anti-mouse IgM secondary antibody, μ-chain specific; Sigma Aldrich Co.) with Red latex beads (LATEX conjugation kit – 400nm Red; Innova Biosciences Ltd.). Before the conjugation procedures, a buffer exchange for the antibody solutions (AbPure Antibody Concentration and Clean-up Kit; Innova Biosciences Ltd.) was performed which removed small interfering substances such as azide and tris from the solutions, and transferred the antibodies to an amine-free buffer (10mM MES buffer at pH 7) that is compatible with the subsequent conjugation procedures. The conjugation was carried out by adding 40μl of the anti-IgG at 0.1mg/ml to the 0.4mg of the pre-treated 400nm Blue polystyrene latex beads. The antibodies covalently bound to the surface of the latex beads via lysine residues during the 15min incubation at room temperature, and the reaction was quenched by adding 1mL of 0.01M tris-buffered saline (TBS) with 0.1% Tween 20. The Blue latex–anti-IgG conjugates were spun at 10,000 rpm for 9 min to remove any of the unbound antibodies, reconstituted to 1% and stored at 4°C until use. The above conjugated steps were repeated for anti-IgM and 400nm Red polystyrene latex beads, yielding 1% red latex–anti-IgM conjugates.

To prepare the conjugate pad that dry-stores the red and blue latex based probes, the probes were first diluted to 0.4% in the conjugate buffer (2mM borate buffer with 5% sucrose), and then used to fully immerse the Glass Fibre Conjugate Pads (EMD Millipore) with 10cm x 5mm dimensions. The conjugate pad was dried at 37°C for 5h and stored until being assembled in to the lateral flow assay.

Assay procedures

In the testing of the two-color lateral flow assay, 10μl of the sample was first dispensed onto the sample pad of the test strip, where the sample may be: rabbit anti-CHIKV IgG (Integrated BioTherapeutics, Inc.), mouse anti-CHIKV IgM (United States Biological), rabbit anti-DENV IgG (LifeSpan BioSciences, Inc.), and/or mouse anti-DENV 4 IgM (antibodies-online). Subsequently 80μl of the chase buffer (1x TBS with 1% BSA, 1.5% Tween 20, 0.1% sodium azide) was dispensed which initiated the sample flow through the length of the test strip. For the duplex IgG/IgM detection for CHIKV (Section 3.2), the anti-CHIKV IgG and IgM samples of 150, 300, 450 and 600μg/ml were prepared by diluting with 1x PBS. For the 4-plex IgG/IgM detection for CHIKV and DENV (Section 3.3), the anti-CHIKV IgG/IgM and anti-DENV IgG/IgM samples at 600 μg/ml in 1x PBS were prepared and used.

Table S.1 Test region red/green/blue/hue intensities at different CHIKV IgG/IgM concentrations

CHIKV IgG/IgM concentration	R intensity*	G intensity*	B intensity*	Hue intensity
0/600	159.32	210.35	250.36	206.36
150/450	169.29	197.84	242.06	216.45
300/300	184.79	200.85	237.46	221.70
450/150	200.41	197.51	231.28	245.15
600/0	204.44	183.71	225.19	269.97

*average values; in range 0-255

Table S.2 Test region red/green/blue/hue intensities for different test strip/regions in 4-plex testing

Test Strip/Region ID		R intensity*	G intensity*	B intensity*	Hue intensity
①	DENV	212.204	204.518	224.618	262.9433
	CHIKV	216.733	201.066	223.519	281.8661
②	DENV	214.554	191.094	240.343	268.5813
	CHIKV	Test region did not develop colors			
③	DENV	Test region did not develop colors			
	CHIKV	213.846	160.614	223.378	290.8878
④	DENV	172.494	223.006	252.575	202.1543
	CHIKV	158.632	218.455	249.947	200.6923
⑤	DENV	164.7	216.083	251.02	204.2843
	CHIKV	Test region did not develop colors			
⑥	DENV	Test region did not develop colors			
	CHIKV	135.397	200.066	245.636	204.8025
⑦	DENV	140.068	193.353	244.441	209.3685
	CHIKV	209.939	175.355	230.87	277.378
⑧	DENV	203.236	177.782	227.576	270.6712
	CHIKV	160.929	203.087	243.918	209.5203
⑩	DENV	211.813	186.983	241.995	267.0814
	CHIKV	165.725	182.562	251.312	228.1966
⑪	DENV	170.395	185.021	241.316	227.6262
	CHIKV	207.526	174.821	233.403	273.4966
⑫	DENV	180.676	182.326	240.405	238.3425
	CHIKV	113.967	198.994	248.89	202.1887

*average values; in range 0-255

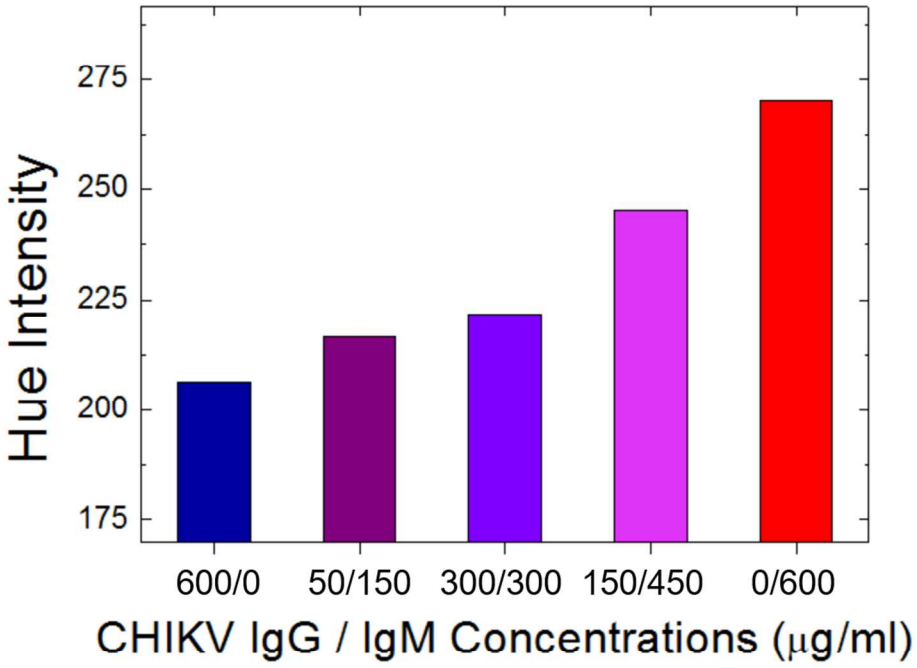

Figure S.1 Test region hue intensity at different CHIKV IgG/IgM concentrations. Hue values are presented in Table S.1

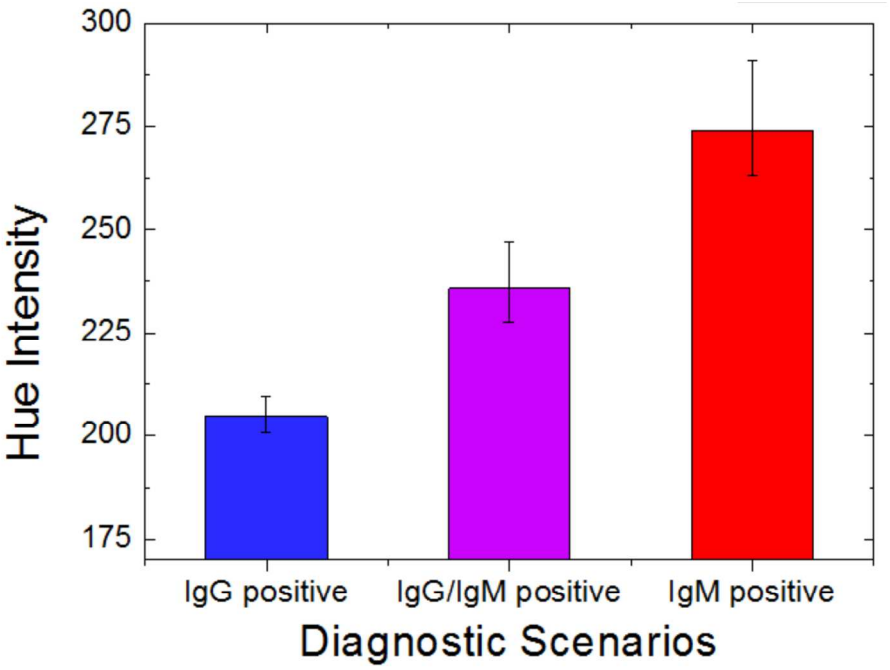

Figure S.2 Hue intensity and associated diagnostic scenarios from 4-plex testing of anti-CHIKV and anti-DENV IgG/IgM. Hue values are presented in Table S.2